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Abstract

This protocol describes a library preparation method for sequencing small RNA. The method uses degenerate adapters to alleviate the problem of bias in the ligation steps during small RNA library preparation, and it optimizes several other parameters to make it appropriate for use specifically with plasma RNA.

Introduction

Extracellular RNAs (exRNAs) have been identified in every biofluid that has been tested. In biofluids, they have been found in extracellular vesicles, ribonucleoprotein complexes, and lipoprotein complexes. ExRNAs are of considerable interest because they can serve as signaling molecules between cells, they have the potential to serve as biomarkers for prediction and diagnosis of disease, and exRNAs or the extracellular particles that carry them might be used for therapeutic purposes.

The Extracellular RNA Communication Consortium (ERCC) is a group of laboratories funded by the U.S. National Institutes of Health. One goal of the ERCC is to develop robust and standardized methods for collecting and processing of biofluids, separating different types of exRNA-containing particles, and isolating and analyzing exRNAs. The "Reference Profiles group":<http://exrna.org/referenceprofiles/> within the consortium is tasked with collecting reliable profiles of the spectrum of extracellular RNAs found in healthy individuals. These reference profiles will serve as benchmarks for comparison with the exRNA profiles from patients with disease. The present protocol for small RNA library preparation was developed by the ERCC Reference Profiles group for use with RNA isolated from human plasma.

Key elements of this small RNA library preparation method include the use of 4 random nucleotides on the end of the adapters ligated to the small RNAs of interest, the use of

higher than usual adapter concentrations, and the use of increased amounts of polyethylene glycol (PEG) in the ligation steps. Small RNA library preparation methods that lack such degenerate adapters have been found to exhibit significant bias in the representation of different RNA sequences.¹⁻⁴ The use of random adapters is designed to alleviate that problem. The use of high adapter concentrations and macromolecular crowding agents such as PEG reduces bias by driving the ligation reactions toward completion. It should be noted that the use of improved ligation conditions and increased adapter concentrations also results in the formation of more adapter dimers, so purification of desired ligation products from such unwanted side products by size fractionation is essential in this protocol.

PCR amplification Small RNA libraries may require 10-20 cycles of PCR amplification depending on the type and amount of input. It has been shown that increased amplification does not significantly affect library bias.^{1,5} Increased number of PCR cycles will, however, increase the amount of adapter dimers that must be separated from the library. Typically, a single PCR and gel purification step is sufficient to remove most of the adapter dimer products from insert-containing PCR products.

Size selection Because of the large excess of adapter dimers in low-input small RNA libraries, electrophoretic purification of PCR products is often necessary. This can be done using either acrylamide or agarose gels, as long as the gel can sufficiently resolve the insert-containing fragments (==150bp) from the adapter dimer fragments (==125bp).

A full list of the protocols developed by the ERCC is available at the "exRNA Portal":<http://exrna.org/resources/protocols/>, the ERCC's website.

This protocol is one of four protocols relating to a 2018 Nature Biotechnology paper.

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"10.1038/protex.2018.049":<http://dx.doi.org/10.1038/protex.2018.049>

Library Preparation for small RNA sequencing using 4N adapters: In house 4N Protocol B

"10.1038/protex.2018.050":<http://dx.doi.org/10.1038/protex.2018.050>

Library Preparation for small RNA sequencing using 4N adapters: In house 4N Protocol C

"10.1038/protex.2018.081":<http://dx.doi.org/10.1038/protex.2018.081/>

Modified TruSeq Small RNA Library Prep using Randomized 4N Adapters: In house 4N Protocol D

"10.1038/protex.2018.052":<http://dx.doi.org/10.1038/protex.2018.052>

Reagents

Oligonucleotide sequences¹

5' adapter (desalted) – 5'

rGrUrUrCrArGrArGrUrUrCrUrArCrArGrUrCrCrGrArCrGrArUrCr(N:25252525)r(N)r(N)r(N)

3' adapter (HPLC purification) – 5' /5rApp/(N:25252525)(N)(N)(N)TGGAATTCTCGGGTGCCAAGG/3ddC/

RT primer (desalted) – 5' GCCTTGGCACCCGAGAATTCCA

RP1 PCR primer (HPLC Purification) – same as Illumina RP1 PCR primer

Indexed PCR primers RPI1-RPI48 (HPLC Purification) – same as Illumina RPI1-RPI48 primers

Reagents for 3' Ligation

T4 RNA ligase 2 truncated KQ (NEB M0373)

10X T4 RNA ligase reaction buffer (included with T4 RNA ligase)

50% PEG 8000 (included with T4 RNA ligase)

RNAseOut RNAse inhibitor (Invitrogen 10777-019)

Strip tubes (Axygen PCR-0208-CP-C or equivalent)

Reagents for adapter depletion

E. coli single-stranded DNA binding protein (SSB) (Promega M3011)

5' deadenylase (NEB M0331)

RecJf (NEB M0264)

Reagents for 5' Ligation

10mM ATP (NEB P0756)

T4 RNA ligase 1 (NEB M0204)

Reagents for Reverse Transcription

Strip tubes (Axygen PCR-0208-CP-C or equivalent)

Superscript III (Invitrogen 18080-044)

5x First strand buffer (included with Superscript III)

0.1M DTT (included with Superscript III)

25mM dNTP mix, diluted 1:2 before use (Thermo Scientific R1121)

RNAseOut RNAse inhibitor (Invitrogen 10777-019)

RiboShredder RNAse blend (Epicentre RS12500)

Reagents for PCR Amplification

NEBNext Ultra II Q5 PCR master mix (NEB M0544) or other high fidelity PCR master mix

DNA Clean and Concentrator 5 columns (Zymo D4004)

Reagents for Gel Purification

5X Novex TBE sample loading buffer (Invitrogen LC6678)

10-well 6% Novex TBE gel (Invitrogen EC6265BOX)

Sybr Gold (Invitrogen S-11494)

Qiagen EB buffer (Qiagen 19086)

SpinX column (Corning 8163)

Reagents for Library Validation

DNA 1000 chip (Agilent, 5067-1504) or High sensitivity DNA chip (Agilent, 5067-4626)

Equipment

Equipment for Ligations and Reverse Transcription

Vacuum concentrator

Thermal cycler

Equipment for PCR Amplification

Thermal cycler or qPCR machine

Equipment for Gel Purification

Thermomixer

Electrophoresis chamber

Equipment for Library Validation

Agilent 2100 Bioanalyzer

Procedure

This protocol is optimized for plasma libraries starting with ~100 μ L of plasma.²

Resuspend the 5' adapter at 25 μ M.

Resuspend the 3' adapter at 10 μ M.

Resuspend the RT primer at 10 μ M.

Resuspend the RP1 PCR primer and the indexed PCR primers (RPI1-48) at 20 μ M.

Make dried PEG strip tubes. These create a high PEG concentration in a small volume for the 3' ligation.

Batches of dried PEG PCR tubes can be made as follows. Add 3 μ L of 50% PEG 8000

(supplied with NEB RNA ligases) into each strip tube and dry in the speedvac using low heat (37°C). This may take 1-2 hours depending on the speedvac. Once dry, the PEG will appear as a white, flaky pellet in the bottom of the tube. Cap the tubes and store desiccated at room temperature. Note that the 50% PEG solution is very viscous, so care must be taken when pipetting. Diluting the 50% PEG to 25% and aliquoting larger volumes may improve pipetting accuracy. Positive displacement pipettes may also be helpful.

3' Ligation

To each strip tube, add:

x μ L RNA

1 μ L Adenylated 3' adapter (10 μ M stock concentration)

5-x μ L Water

6 μ L Total

The dried PEG in the strip tube will take up about 1 μ L in the reaction after resuspension.

1. Heat tube containing RNA and adapter to 70°C for 2 minutes, then snap cool on ice.

To each tube containing 7 μ L of denatured RNA and adapter, add:

1 μ L 10X T4 RNA ligase reaction buffer

1 μ L RNaseOut RNase inhibitor

1 μ L T4 RNA ligase 2 truncated KQ

3 μ L Total

2. Incubate at 25°C for 2 hours.

3. Add 1 μg *E. coli* SSB (diluted in 1X ligase buffer) and incubate at 25°C for 10 minutes.
4. Add 1 μL 5' deadenylase and incubate at 30°C for 1 hour.
5. Add 1 μL of RecJf and incubate at 37°C for 1 hour.

5' ligation

In a separate tube, add 1 μL of 5' adapter (25 μM stock concentration) per ligation and denature at 70°C for 2 minutes, then snap cool on ice.

To the tube of denatured 5' adapter, add (per ligation):

1 μL 10mM ATP

1 μL T4 RNA ligase 1

1. Add 3 μL of adapter, ATP and ligase mix to the completed 3' ligation for a total volume of 16 μL .
2. Incubate at 25°C for 1 hour.

Reverse transcription (RT)

To a new tube, add:

1 μL RT primer (10 μM stock concentration)

6 μL Ligated RNA (Store leftover ligated RNA at -70°C.)

7 μL Total

1. Heat to 70°C for 2 min, then snap cool on ice.

Add:

2 μ L 5x First strand buffer

0.5 μ L 12.5 mM dNTP mix

1 μ L DTT

1 μ L RNaseOut

1 μ L Superscript III

5.5 μ L Total

Add to 7 μ L denatured RNA/primer for 12.5 μ L total RT reaction.

2. Incubate at 55°C for 1 hour, then at 70°C for 15 minutes.

3. Add 1 μ L RiboShredder RNase blend and incubate at 37°C for 15 minutes.³

PCR amplification

25 μ L PCR master mix (NEBNext Ultra II Q5 or equivalent)

2 μ L Illumina RP1

2 μ L Illumina RPI1-48 reverse index primer

8 μ L water

13 μ L cDNA

50 μ L Total

1. Amplify for 15-20 cycles using the following program:

98°C 30 sec. 1X

98°C 10 sec.

60°C 30 sec. 15-20X

65°C 35 sec.

65°C 2 min. 1X

2. Purify PCR product using DNA Clean and Concentrator 5 columns. Elute in 50 µl (or a smaller amount if desired).

Gel purification

1. Add 12.5 µL 5X Novex TBE sample loading buffer to PCR product.
2. Split into 3 lanes on a 10-well 6% TBE gel (Invitrogen EC6265).
3. Run for ~80 minutes at 100 volts.
4. Stain with Sybr Gold (Invitrogen S-11494) and cut out band at ~149bp.
5. Crush gel slice (i.e. in Gel-breaker tube) and soak fragments overnight in 300 µL Qiagen EB buffer at room temperature in Thermomixer.
6. Spin through SpinX column (Corning 8163) to remove gel fragments and ethanol precipitate DNA.
7. Resuspend in 10-15 µL and run on Bioanalyzer DNA 1000 chip to check library size.

Troubleshooting

1) Oligonucleotide sequences © 2016 Illumina, Inc. All rights reserved. Derivative works created by Illumina customers are authorized for use with Illumina instruments and products only. All other uses are strictly prohibited.

2) This protocol is optimized for plasma libraries starting with ~100 µL of plasma. Libraries with more starting material can probably tolerate higher adapter concentration and higher PEG concentration. Libraries with less starting material may need reduced adapter or PEG concentration. **Caution:** Differential expression analysis, i.e. comparison of RNA levels across samples, should not be done between data sets from libraries

prepared using different PEG or adaptor concentrations. Relative read numbers are affected by these parameters.

3) Incubating the reverse transcription reaction with RiboShredder RNase blend might not be necessary.

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